SELF-ASSEMBLING COMPLEXES FOR GENE DELIVERY

FROM LABORATORY TO CLINICAL TRIAL

Edited by

Alexander V. Kabanov
University of Nebraska, Omaha, USA

Philip L. Felgner
Vical, Inc., San Diego, USA

Leonard W. Seymour
CRC Institute for Cancer Studies, Birmingham, UK

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1 Polymorphism of lipids, nucleic acids, and their interactions

D.D. Lasic, D. Papahadjopoulos and R. Podgornik

Introduction

While 'nonliving' nature creates large and well-ordered systems, such as monocrystals some meters long which, however, are characterized by a rather simple structure, the 'living' nature creates much more complex structures of relatively complicated (macro)molecules. These include self-assembly of lipid molecules into structures with complex topology and the ability to compartmentalize space (Lasic, 1993; Evans and Wennerstrom, 1994; Lipowsky and Sackmann, 1995; Lasic and Barenholz, 1996a,b). Another example is molecules of nucleic acids that can store and duplicate information and also use it for spatially and temporally controlled function, molecular construction, and action (Bloomfield *et al.*, 1974; Saenger, 1984; Watson *et al.*, 1983). The functioning of living systems is based not only on the order, organization, and accompanying information, but also requires rapid diffusion of this information and molecules supporting it between various compartments and through barriers separating them. Therefore, the organization in living systems is based on liquid crystalline rather than on crystalline systems where the diffusion is too slow to support biological systems.

Lipid bilayers and other lipid phases as well as DNA and its liquid crystalline structures are the best-known examples of supermolecular and supramolecular organization in biology. However, we should not forget that many other molecules, molecular aggregates, or cells in living tissues actually form and function as liquid crystalline phases. In addition to the structural and dynamic information of lipid bilayers, DNA itself possesses not only external conformational information but also internal genetic information encoded in the sequence of bases. The aim of molecular biotechnology is to tailor this genetic information *in vitro* and express the genes in exogenous cells and organisms.

In recombinant DNA technologies and genetic engineering nucleic acids are introduced into cells by physical methods (e.g. electroporation) or simple chemical

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treatment (e.g. calcium–phosphate precipitation). The delivery of genes into appropriate cells *in vivo* in gene therapy involves more complex techniques, which at present use mostly synthetic viral constructs or lipid-based systems. Among micelles, mixed micelles, emulsions, and liposomes, the latter have shown by far the most promise. In order to discuss the interaction of liposomes with DNA and the delivery of these complexes into cells, we shall briefly review the polymorphic behavior of each system and their mutual interactions.

Polar lipids are molecules containing a hydrophilic and hydrophobic part. Therefore, these molecules form ordered structures in aqueous solutions. Lipids give rise to structurally very rich phase diagrams, although it seems that in living systems predominantly lamellar and to a much lesser extent hexagonal and cubic phases, along with isotropic micellar phases, are present. For instance, cell membrane is actually a piece of a fully swollen lamellar phase self-closed into a spherical bag (with various distortions), which contains numerous proteins associated with it to carry their function. The richness of different phases formed by lipids depends on molecular geometry and interaction properties of lipid molecules.

Similarly, DNA molecules organize into highly ordered structures at higher concentrations or upon interactions with particular agents. This normally results in various forms of condensed DNA in which the hydrodynamic molecular volume can be reduced up to a millionfold if compared to the DNA volume in solution. The structural diversity of DNA mesophases is determined by the stiffness of the DNA molecule, strong interactions between opposed duplexes in condensed phases, effects of the phosphate backbone charges as well as much weaker, and as yet poorly understood, chiral interactions characteristic of its double helix form. While there are no strong attractive interactions between negatively charged nucleic acids and negative or neutral liposomes, there are strong interactions with positively charged liposomes. They can be used to complex DNA and transfer it into appropriate cells *in vitro* or *in vivo*.

Lipid polymorphism

Lipid molecules organize into ordered structures to maximize hydrophilic and minimize hydrophobic interactions (Tanford, 1980). The solvation of the polar head groups and dispersive interactions of the hydrocarbon chains of the lipid molecules provide for the negative contribution to the free energy during formation of those structures. The positive contributions to the free energy result from the repulsive interactions of the polar head groups, including steric and electrostatic repulsion. The hydrophobic effect, i.e. the increase in entropy of the solvent during segregation of the polar and nonpolar groups is the fourth major factor causing ordering and self-assembly. As a result lipid molecules self-assemble into different microscopic structures, such as bilayers and micelles (spherical, rod-like or disk-like), which, especially at higher concentrations, can further pack into macroscopically ordered phases, such as lamellar, hexagonal and cubic. In addition,

the morphology of these macroscopic phases depends on interparticle interactions, which can be repulsive or attractive. The latter ones are van der Waals and ion correlation attraction, while the major repulsive interactions are electrostatic, steric, hydration, undulation, and protrusion forces (Israelachvili, 1985). These amphiphilic systems can be macroscopically homogeneous and continuous (often referred to as lyotropic liquid crystals), or can be dispersed into colloidal suspensions. Typically such suspensions are macroscopically isotropic but contain local order and organization. In general, a tensorial order parameter describes their structure (de Gennes and Prost, 1993).

A useful parameter to describe the shape of the repeating units in liquid crystalline phases or macroscopically isotropic solutions of colloidal particles is the so-called packing parameter (Israelachvili, 1985). Briefly, in the cases where the cross-section of the hydrophobic part (b) is similar to the area of the polar head (a) of the lipid, these molecules pack into flat bilayers with zero curvature ($c = 0$). At $b > a$ inverse structures with negative curvature form, such as inverse micelles and inverse hexagonal II phase. At $a > b$ normal micelles ($c > 0$) are observed, which can form isotropic phases or pack into liquid crystalline phases, such as hexagonal I phase. Cubic phases are typically different three-dimensional (3D) networks of minimal surfaces defined by zero mean curvature.

Figure 1.1 shows the morphology of various lipid phases and particles. These are mostly thermodynamically stable structures, which can form macroscopically ordered liquid crystalline phases or stable colloidal dispersions. The morphology of these phases changes with lipid concentration and temperature. Low temperature phases are normally lamellar in which tilted crystalline phase (L_c or ripple phase P_β) or nontilted (L_β and $L_{\beta'}$) lipid bilayers form 3D, two-dimensional (2D), or one-dimensional (1D) crystalline or gel phases. Terminology from thermotropic liquid crystals can be also used. Briefly, these phases are smectic, and SmA describes 2D fluid with no tilt while a variety of SmC phases with different indices encompass tilted phases with different degrees of 2D order. In these phases, hydrocarbon chains of lipid molecules are frozen. Upon melting, liquid crystalline phases with 1D (lamellar L_α), 2D (hexagonal H, or 3D (cubic) positional order can form. The most frequently formed phases are micellar, lamellar and hexagonal. Normal hexagonal phase consists of long cylindrical micelles ordered in a hexagonal array, while in the inverse hexagonal II phase water channels of inverse micelles are packed hexagonally. In excess water, such arrays are coated with a lipid monolayer. The morphology of these phases can be maintained upon their (mechanical) dispersal into colloidal dispersions. Despite the fact that energy has to be used to generate dispersed mesophases, relatively stable colloidal dispersions of particles with lamellar, hexagonal, or cubic symmetry can be formed.

In the presence of a water-immiscible organic phase emulsion droplets can assemble. In regions of phase diagram which are rich in water, oil-in-water emulsions and microemulsions ($c > 0$) can be formed. In contrast, in oil-rich regions these spherical particles have negative curvature and are, therefore, water-in-oil emulsions. The intermediate phase between the two is a bicontinuous

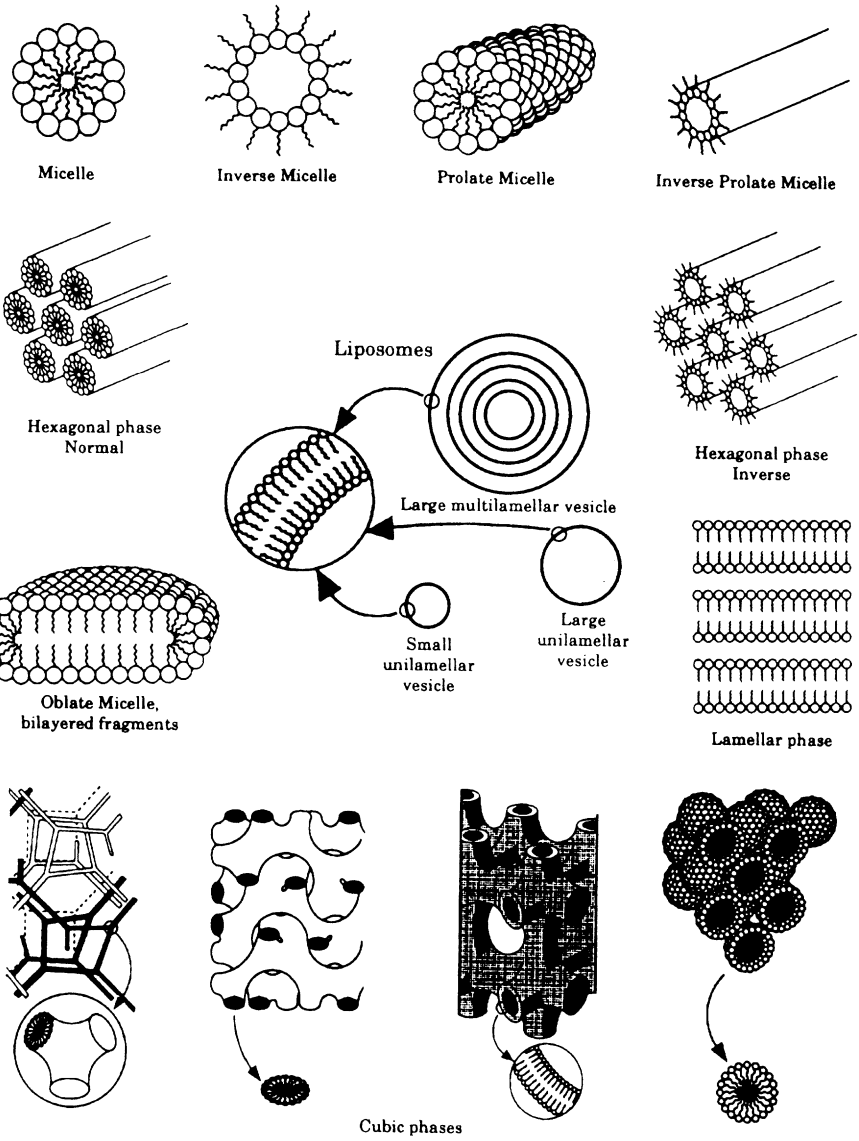

Figure 1.1 Lipid polymorphism. In aqueous solutions lipid molecules self-assemble into various structures which can organize macroscopically into different phases or form isotropic colloidal solutions. Various types of liposomes are shown in the middle of the figure. Large multilamellar vesicles can have hundreds of concentric bilayers and can be up to millimeters in dimensions, while large unilamellar vesicles are typically in the size range of 100–1000 nm. The thickness of the membrane is typically 4 nm.

emulsion, which has zero average curvature. As will be discussed for liposomes, only microemulsions can form spontaneously (analogously to micelle formation) while for the formation of a homogeneous emulsions some energy has to be dissipated into the system.

Fine structure of these phases, as well as the size and shape of colloidal particles, is probably determined by the molecular geometry of lipid molecules, their aqueous solubility and effective charge, as well as weaker interactions, such as intramolecular and intermolecular hydrogen bonds, stereoisomerism, and interactions within the medium. Obviously, these phases depend on the temperature, lipid concentration, and electrostatic and electrodynamic interactions with the solvent and solutes. In the case of charged lipids, the effects of counterions — especially anions, may also be important. Ionotropic transitions have been observed with negatively charged phospholipids in the presence of metal ions leading to aggregation and fusion (Papahadjopoulos *et al.*, 1990). In cationic amphiphiles it was shown that simple exchange of counterions could induce micelle–vesicle transition (Talmon *et al.*, 1983). Lipid polymorphism is very rich and even single component lipid systems can form a variety of other phases, including ribbon-like phases, coexisting regions, and various stacks of micelles of different shapes (Luzzati *et al.*, 1960).

As mentioned above, lipid polymorphism includes not only various phases as a function of composition, but also phase transitions as a function of temperature, pressure, ionic strength, and counterions (Duzgunes *et al.*, 1983; Straubinger *et al.*, 1983). Since lipid bilayers, either in lamellar phase or as liposome membranes, are the most important phase in current applications we shall briefly review phase changes of lipid bilayers. At very low temperatures a 3D crystal exists in which tilted lipid bilayers are stacked together. Upon heating, various rearrangements in the 2D crystalline bilayers start to happen, including loss of 2D order (L_c) and tilt (L_β). With a further increase in the temperature at the gel–liquid crystal phase transition the untilted or rippled bilayer (P_β phase) changes into a bilayer membrane with disordered 2D polar heads and fluid hydrocarbon chains, termed

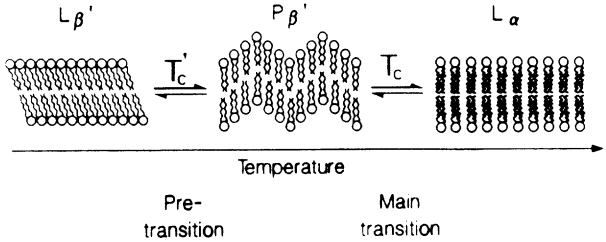

Figure 1.2 Main phase transitions in the lipid bilayer. Liposomes are typically in disordered fluid phase (L_α). Below the phase transition temperature, the fluid hydrocarbon chains freeze resulting in the formation of so-called ripple phase (P_β') and solid ordered bilayers (L_β'). Addition of cholesterol increases the order in fluid (disordered) phase and reduces the order in the solid (ordered) phase. Above a certain critical concentration of cholesterol (33.3 mol% for lecithin bilayers) the phase transition is abolished and the system is in a fluid phase in a wide temperature range.

L_α phase (Figure 1.2). Liposome bilayers can also undergo such phase transition. Electron microscopy has revealed fluid phase, rippled, and crystalline phases in which spherical liposomes transform into polyhedral liposomes due to very high values of bending elasticity of crystallized bilayers. (Lasic, 1993; Frederik, 1996). Figure 1.3 shows freeze fracture electron micrographs of liposomes in fluid, ripple, and solid phases.

The isotropic colloidal solutions of self-assembled lipid particles are most frequently used for complexing with nucleic acids in medical applications. Therefore, we shall mainly concentrate on micelles, mixed micelles, and liposomes. The macroscopic lamellar phase is probably not of interest for gene delivery, while inverse micellar and inverse hexagonal phases may be important in various bilayer interactions, such as fusion.

In contrast to liquid crystalline phases and micellar solutions, liposomes are in general not a thermodynamically stable phase but rather a kinetically trapped system. This conclusion can be easily inferred from the fact that energy in the form of ultrasound, extrusion (pressure), homogenization, or other forms of mechanical or chemical energy has to be used to prepare liposomes. However, in the case of highly charged and asymmetric bilayers in low ionic strength media, thermodynamically stable liposomes can form spontaneously (Gebicki and Hicks, 1973; Hargreaves and Deamer, 1978; Lasic, 1988; Lasic *et al.*, 1988; Kaler *et al.*, 1989). Typically, these spontaneously formed liposomes are too heterogeneous to be practically useful. However, by carefully balancing electrostatic and steric interactions, extremely homogeneous spontaneous vesicles have been prepared recently (Joannic *et al.*, 1997). In this system, the spontaneous breaking of symmetry of the density of the surface-attached polymer between the two leaflets of the bilayer causes nonzero spontaneous curvature. On the other hand, infinite swelling of lipid bilayers is known to produce a large fraction of small unilamellar vesicles (Hauser *et al.*, 1986). Accordingly, swelling of cationic lipids in low ionic strength aqueous media with simple agitation already produces mostly unilamellar and few-lamellar liposomes in the size range 300–600 nm. In certain cases simple vortexing of swelling lipid films can be used to prepare cationic liposomes for DNA condensation and transfection. Numerous vesicles generated in living cells are also created by the input of chemical energy indicating that life itself, from a lipid bilayer prospective, is an irreversible and energy driven process.

While lamellar phases can be dispersed into liposomes, the hexagonal and cubic phases can be dispersed into stable colloidal suspensions of smaller fragments of these phases termed 'hexasomes' and 'cubosomes.' However, utility of those structures in drug and gene delivery remains to be determined.

Generally speaking, since vesicles are not in a thermodynamically stable state we cannot calculate their size distribution like those of micelles or microemulsion droplets, for instance. Nevertheless, we can express some other properties of liposomes using various thermodynamic functions. These properties include specific heat of phase transitions, lateral compressibility, and bending modulus. All

Figure 1.3 Electron micrographs of liposomes in fluid (A), ripple (B), and solid (C) phase. Upon lowering of temperature, the bending elasticity of bilayers increases and the spherical shape (A) changes into polyhedral (C), because the bilayers are too rigid to curve into a sphere. An intermediate phase contains ripples and was also observed in finite lipid particles, such as liposomes (B). For the fluid liposomes multilamellar vesicles of egg phosphatidylcholine are shown (A) while the other two structures (B, C) are large unilamellar liposomes from dipalmitoyl phosphatidylcholine.

this simply means is that liposomes are objects, which are characterized by specific finite-size effects.

Liposomes can be prepared by a variety of techniques (Bangham, 1983; Lasic, 1993). For large-scale applications, extrusion of large multilamellar vesicles prepared by hydration of lipids or homogenization are mostly used. The first method yields liposomes with easily controllable size distributions (Olson *et al.*, 1979) while the latter is used for the preparation of smaller liposomes (Mayhew *et al.*, 1984). Hydration can proceed from dried lipid films, lyophilized cakes (typically from tertiary butanol), or spray-dried lipid powder, as well as by injection of lipid solutions in ethanol or propylene glycol into the aqueous phase followed by dialysis to remove the organic solvent (Lasic, 1993).

Liposomes, which can be small (S), large (L), or giant (G) unilamellar (U), oligolamellar (O), or multilamellar (ML) vesicles, are not the only colloidal form of bilayer-forming lipids in aqueous suspensions. In some systems, such as phosphatidylserine aqueous mixtures in the presence of Ca^{2+}, a different structure was observed — cochleae cylinders, which are rolls of larger lipid bilayers (Papahadjopoulos *et al.*, 1975). These particles have found applications in drug and gene delivery. In many synthetic lipid systems similar structures, termed lipid tubules, were observed (Spector *et al.*, 1996). These structures are actually self-closed cylinders, open at the ends. It appears that such 2D continuous structures are favored when (chiral) bilayers freeze and molecules tilt in the bilayers, because tilted bilayers cannot self-close into a spherical particle without significant defects, which are energetically unfavorable compared to the open ends of the cylinders. Polymorphism of colloidal lipid particle morphology also includes multi-compartment liposomes (microscopic globules of foam), micelles of various shapes and suspensions of dispersed lamellar and hexagonal phases.

Similarly to the thermodynamic phases as discussed above, liposomes can undergo several phase transitions and this can be used to prepare liposomes with specific properties. Temperature-sensitive liposomes, for instance, greatly increase membrane permeability at T_c (Papahadjopoulos, 1978). Lamellar to hexagonal II phase transition can be used to trigger the release of encapsulated molecules or to induce fusion. Typically, such bilayers contain dioleoyl phosphatidylethanolamine (DOPE), which has a tendency to form hexagonal II phase ($b > a$), mixed with a bilayer-forming lipid ($a = b$ or $a > b$) so that $\langle a \rangle = \langle b \rangle$, where the angled brackets designate an average value in a bilayer. Upon depletion of the latter lipid, either by dissociation or cleavage of part of its polar head, bilayers become unstable and liposomes break down. Lamellar to micellar phase transition results in liposome disintegration and can be induced by hydrolysis of fatty acid chains. Let us define an average packing parameter for the mixed bilayer as $\langle P \rangle = \Sigma_i P_i x_i$, where x_i is the molar fraction of lipid i with a specific packing parameter $P_i = b_i/a_i$. Then for the lamellar to micellar transition $\langle P \rangle$ is decreasing while for lamellar to hexagonal transition $\langle P \rangle$ is increasing compared to $\langle P \rangle = 1$ that characterizes a stable lamellar phase.

Applications of lipids in gene delivery have relied mostly on the use of liposomes composed of cationic lipids. Some researchers have used simple and mixed micelles and emulsions, while no data on hexasomes and cubosomes have been published. We believe that this is due to the fact that cationic liposomes provide the best lipid dispersal in a metastable bilayer structure which seems to be the most suitable to induce stable DNA condensation and its protection.

Polymorphism of nucleic acids

We can define several levels of DNA organization similarly to proteins (Bloomfield *et al.*, 1974; Watson *et al.*, 1983). Primary structure encompasses the sequence of bases. Secondary structure describes the structure of the molecule. Tertiary structure describes the conformation of DNA in solution. Normally, secondary structure of DNA is a double helix, which can exist in several conformations. In solution, the B structure is predominant. In the canonical form of this structure (Saenger, 1984), the bases are perpendicular to the axis of the molecule and are 0.34 nm apart, and 10 of them make one turn of the helix. These parameters can vary for DNA in solution where up to 10.5 base pairs can make a whole turn of the helix (Sinden, 1994). In the A structure, the bases are tilted with respect to the direction of the helix and this arrangement yields an internal hole, a wider diameter and, closer packing. Other conformations, such as the left-handed Z form, are much more rare.

In isotropic solutions, DNA can be in one of several forms. Linear molecules are typically straight on the order of a persistence length (defined as the length of the molecule containing kT of elastic energy if bent in a circle with the same radius) while for longer lengths they form a worm-like random coil. DNA can also exist in a circular form. The torsion in this conformation is normally so large that it induces a break in one strand forming so-called 'nicked' DNA. The most frequent DNA conformation in solution, however, is the supercoiled state. It does not form only in the combination with proteins, but also occurs in free DNA molecules, which are frequently under- or over-wound. Closed circular DNA has, due to peculiarities of its synthesis, a constant number of the (interwinding) crossings resulting in topological constraints, which can be relaxed only by breaking the double helix. This is a general phenomenon with important biological consequences. It seems that free energy of negative supercoiling catalyzes processes such as DNA replication and transcription which rely on DNA unwinding (Boles *et al.*, 1990). While the sequence of bases in introns determine the nature of the proteins synthesized, it is not impossible that such structural features in noncoding regions dictate the temporal and spatial evolution of DNA encoded information.

DNA is a macromolecule of macroscopic dimensions. For instance, the human genome is coded in approximately 3 billion base pairs, which give rise to an approximately 1 m-long molecule. Obviously, this molecule has to undergo several compaction processes in order to fit in an approximately 6 μm cell nucleus. In the natural environment, basic proteins pack DNA in chromatin structures in which DNA is organized into well-ordered arrays. *In vitro*, DNA can be packed into very

tight and dense structures as well, and we shall introduce below various agents which can induce DNA packaging. Addition of those agents typically induces DNA condensation, that is a random coil–globule transition (Bloomfield, 1996). DNA condensation *in vitro* was observed relatively early. It was found that multivalent ions, such as spermidine^{3+}, spermine^{4+}, $Co(NH_3)^{3+}$, polylysine, histones, etc. can condense DNA, resulting in either rod-like or toroidal condensates (Bloomfield 1996). In these studies it was shown that large DNA molecules could become condensed not only by cations, which shield the repulsion of the negative charges, but also by neutral polymers which deplete water and crowd DNA into small pockets osmotically (Podgornik *et al.*, 1995). Typically, the use of polyethylene glycol (PEG) and ethanol solution is the easiest way to condense DNA. The osmotic pressure from the PEG polymer and the reduced activity of water, due to the presence of ethanol, drive the DNA condensation. For a general introduction to DNA mesophases see the proceedings of the international interdisciplinary workshop 'Structure and function of DNA – a physical approach' (Abbaye du Mont Saint-Odille, 9/30–10/5, 1996).

Like lipids, DNA, being a primary example of a biological polyelectrolyte (Oosawa, 1971), forms highly ordered mesophases such as lyotropic liquid crystals once its concentration is raised above a critical value (Rill *et al.*, 1991). Figure 1.4 shows various liquid crystalline phases of DNA, which will be discussed below. But contrary to the case of lipid mesophases, where the shape of constituent molecules plays a determining role, the organization of DNA in condensed phases is primarily a consequence of its relatively large stiffness (Livolant and Leforestier, 1997). The stiffness of a polymeric chain expressed in terms of its persistence length for DNA amounts to about 500 Å (Bloomfield *et al.*, 1974). For example, hyaluronic acid, another ubiquitous biological polyelectrolyte (Elias, 1984), which is far less stiff than DNA (persistence length about 10 Å) makes no ordered mesophases. The orientational ordering of DNA at high concentrations is promoted mostly by the interplay between entropically favored disorder or misalignment and the consequent price in terms of the high interaction energy. The mechanism of orientational ordering is, therefore, the same as in standard short nematogens (de Gennes and Prost, 1993). The main difference being due to the large length of polymeric chains. The discussion that follows will concentrate on very long, the order of 1000 persistence lengths, DNAs.

Ordering of DNA can be induced by two alternative mechanisms. First of all attractive interactions between different DNA segments can be enhanced by adding multivalent counterions that promote either counterion correlation forces, as was first suggested by Oosawa (1971), or long-range hydration attraction (Rau and Parsegian, 1992). Further, addition of alcohol to the bathing solution also results in decreasing the electrostatic repulsion of DNA segments (Bloomfield, 1996). In these cases DNA aggregates spontaneously. Alternatively, one can add neutral crowding polymers to the bathing solution that phase separate from DNA and exert osmotic stress on the DNA subphase (Podgornik *et al.*, 1995). In this case the intersegment repulsions in DNA are simply counteracted by the large externally

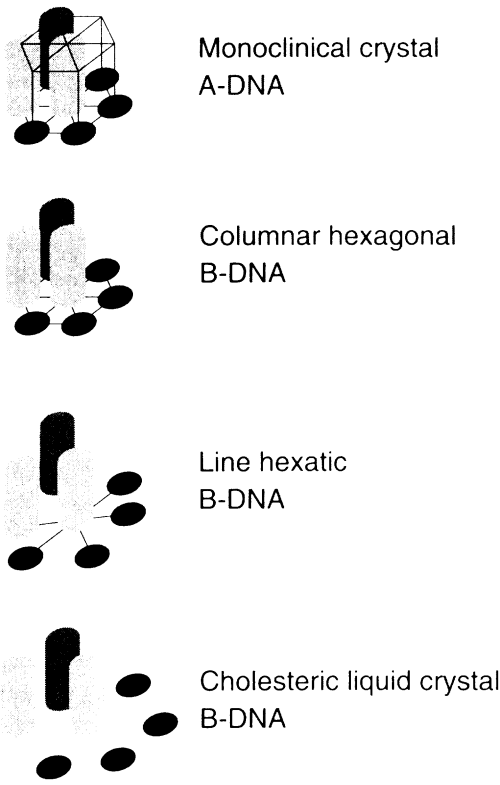

Figure 1.4 A sequence of DNA mesophases from higher to lower density. Only A-DNA can be in a crystalline form of a monoclinic symmetry. B-DNA can be found in all the lower density mesophases, i.e. liquid crystalline phases. Although columnar hexagonal DNA mesophase has not been observed with long DNA, it could exist at the upper end of the line hexatic line. Black lines show the long-range order in different directions.

applied osmotic pressure and the DNA is forced to condense under externally imposed constraints. This is somewhat analogous to a Boyle experiment but with osmotic pressure playing the role of regular pressure. The main difference being that regular pressure is set mechanically while osmotic pressure has to be set through the chemical potential of water, which is in turn controlled by the amount of neutral crowding polymers (such as PEG, polyvinyl pyrollidone, or dextran) in the bathing solution (Parsegian *et al.*, 1986).

At very high DNA densities, where the osmotic pressure exceeds 160 atm, DNA can exist only in a (poly)crystalline state (Lindsay *et al.*, 1988). Nearest neighbors in such an array are all oriented in parallel and show correlated (nucleotide) base stacking between neighboring duplexes. This means that there is a long-range correlation in the positions of the backbone phosphates between different DNA molecules in the crystal. The local symmetry of the lattice is monoclinic. Owing to

the high osmotic pressure, DNA is actually forced to be in an A conformation characterized by a somewhat larger outer diameter as well as a somewhat smaller pitch than in the canonical B conformation, which persists at smaller densities. If the osmotic pressure of such a crystal is increased above 400 atm, the helix begins to crack and the sample loses structural homogeneity (Lindsay *et al.*, 1988).

On the other hand, lowering the osmotic pressure does not have a pronounced effect on the DNA crystal until we reach a limit of 160 atm. Then the crystal as a whole simultaneously expands while individual DNA molecules undergo an A–B conformational transition (Lindsay *et al.*, 1988). This phase transformation is then first order and, besides being a conformational transition for single DNAs, it is connected with the melting of the base stacking as well as positional order of the helices in the lattice. The ensuing low-density mesophase, where DNA is in the B conformation, is characterized by a short-range base stacking order, short-range 2D positional order, and a long-range bond orientational order (Podgornik *et al.*, 1996). This order is connected with the spatial direction of the nearest neighbors (Strandburg, 1991). It is for this reason that this phase has been termed line hexatic phase (or hexatics)—usually arising only in 2D systems—being characterized by long-range bond orientational order. It is also possible that there is a hexatic–hexagonal columnar transition somewhere along the hexatic line, although direct experimental proof is lacking at this point. The difference between the two phases is that the hexagonal columnar phase also has a long-range positional order, so it is a real 2D crystal (see Figure 1.4) (Durand *et al.*, 1992). It is the long-range bond orientational order that gives the line hexatic phase some crystalline character (Harris *et al.*, 1977). The DNA duplexes are still packed in parallel, while the local symmetry perpendicular to the long axes of the molecules is changed to hexagonal. The directions of the nearest neighbors persist through macroscopic dimensions (on the order of mm) while their positions already tend to become disordered after several (typically from five to 10) lattice spacings. This mesophase has a characteristic X-ray scattering fingerprint. If the X-ray beam is directed parallel to the long axis of the molecules it will show an hexagonally symmetric diffraction pattern of broad liquid-like peaks (Chaikin and Lubensky, 1995).

Typical lattice spacing in the line hexatic phase are between 20 and 35 Å (or equivalently between 600 and 300 mg/ml of DNA) (Podgornik *et al.*, 1996). The large repulsion forces stemming from the removal of water from the phosphates of the DNA backbone mostly determine the free energy in this mesophase. Hydration forces (Rau *et al.*, 1984) at these large densities are typically independent of the ionic strength of the bathing solution, depend exponentially on the interhelical separation, and decay with a decay length of about 3 Å (Leikin *et al.*, 1993).

When the osmotic pressure is lowered to approximately 10 atm (corresponding to interaxial spacing of about 35 Å or a DNA density of about 300 mg/ml) the characteristic hexagonal X-ray diffraction fingerprint of the line hexatic mesophase disappears continuously. This suggests a continuous second order transition into a low-density cholesteric phase (Podgornik *et al.*, 1996). This phase is characterized by a short-range (or effectively no range) base stacking order,

short-range positional order, short-range bond orientational order, but long-range cholesteric order, manifested in a continuing rotation of the long axis of the molecules in a preferred direction. In this sense the cholesteric DNA mesophase would retain the symmetry of a 1D crystal. The X-ray diffraction pattern of the DNA in the cholesteric phase is isotropic and has the form of a ring. Crossed polarizers, however, reveal the existence of a long-range cholesteric order as in short chiral molecules (Podgornik *et al.*, 1995). It is only at this low density that the chiral character of the DNA finally makes an impact on the symmetry of the mesophase. It is not yet fully understood why the chiral order is effectively screened from the high-density DNA mesophases (Harris *et al.*, 1977).

The free energy of the DNA cholesteric mesophase appears to be dominated by the large, elastic shape fluctuations of the DNA molecules that leave their imprint in the very broad X-ray diffraction peak (Strey *et al.*, 1998). Instead of showing the expected exponential decay characteristic of screened electrostatic interactions (Frank-Kamenetskii *et al.*, 1987), where the decay length is equal to the Debye length, it shows a fluctuation-enhanced repulsion similar to the Helfrich force existing in the flexible smectic multilamellar arrays (Lipowsky, 1995). The action of the fluctuations is twofold: they boost the magnitude of the existing screened electrostatic repulsion while at the same time extending its range through a modified decay length equal to four times the Debye length. This enhancement in the range of the repulsive force is a consequence of the coupling between the bare electrostatic repulsions of exponential type and the elastic shape fluctuations, described through elastic curvature energy proportional to the square of the second derivative of the local helix position (Podgornik and Parsegian, 1990; Strey *et al.*, 1998). The similarity of the free energy behavior of the smectic arrays with repulsive interactions of Helfrich type and the DNA arrays in the cholesteric phase, that can be understood in the framework of the Helfrich-type enhanced repulsion, represents a consistency test for our understanding of flexible supermolecular arrays.

With DNA at still smaller densities the predominance of the chiral interactions in the behavior of the systems are preserved. Recent work on the behavior of low-density DNA mesophases indicates that the cholesteric part of the phase diagram might end with a sequence of blue phases, emerging as a result of the loosened packing constraints coupled to the chiral character of the DNA molecule (Leforestier and Livolant, 1994). At a DNA density of about 10 mg/ml, the cholesteric phase line would end with DNA reentering the isotropic liquid solution where it remains at all subsequent densities, except perhaps at very small ionic strengths (Wang and Bloomfield, 1991).

As already noted, DNA can be condensed efficiently in solution with multivalent cations, alcohol, basic proteins, or cationic liposomes (Bloomfield, 1996). In all these cases, however, aside from inducing the condensation of DNA, the condensing agents also effectively perturb the mesoscopic as well as macroscopic structure of the ensuing condensed phases (Bloomfield, 1991). Therefore, multivalent cations would tend to organize condensed mesophases into macroscopic

aggregates of a toroidal shape, while alcohols at low volume percent would tend to favor rod-like macroscopic aggregates. However, neither polyvalent cations nor alcohols significantly perturb the microscopic structure of the condensed DNA solution (Bloomfield, 1996). It is still locally hexagonally packed and has the same free energy as if condensed exclusively by counteracting osmotic pressure. The situation in the case of condensation by cationic liposomes is very much different.

Interactions of micelles and liposomes with nucleic acids

Thermodynamic parameters can quantitatively explain the behavior of simple solutions and solids. However, both isotropic solutions of DNA and liposomes are colloidal dispersions and, therefore, during their interaction, kinetic factors are, in addition to thermodynamics, also very important. While there are not many interactions between DNA and neutral or anionic liposomes, interaction with cationic liposomes changes the morphology of both reactants.

We have characterized the DNA cationic liposome interactions, and the behavior of the structure of the resulting semi-solid colloidal particles, as a function of thermodynamic parameters, such as concentration, temperature, ionic strength, surface charge (pK), and pH. Phase diagrams of DNA–lipid complexes in the phase space of DNA and cationic liposome concentration were determined (Lasic, 1997). As expected, higher reactant concentrations and ionic strengths resulted in more precipitation. Additionally, however, a strong kinetic component has been noticed. It was found that the kinetics of mixing, as well as the order of mixing of the two solutions, could give rise to irreversible and path-dependent flocculation or precipitation. Qualitative explanations as to the rate and order of mixing have been postulated. Briefly, in the phase diagram DNA–cationic liposome precipitation occurs around the electrical neutrality diagonal. To prepare stable complexes on either side of the diagonal, mixing should be performed in such a way that the solubility gap should not be crossed. This means that the minor component, with respect to the charge, always has to be added into the major one. Also, it was shown that quick dispersal assures the least precipitation. This was qualitatively explained by the formation of a very large number ('sudden burst') of small crystallization/nucleation embryo which give rise to many small complexes as opposed to growth closer to the thermodynamic equilibrium which results in the formation of fewer larger particles (Lasic, 1997).

A very interesting observation was that an excess of DNA solution can dissolve multilamellar vesicles, i.e. upon adding DNA into a turbid solution of large multilamellar (or oligolamellar) vesicles the turbidity disappears. This probably indicates that DNA can dissolve and disintegrate large liposomes. The structure of such solutions has not yet been determined. Preliminary analyses point to DNA helices (partially) coated by lipid tubes. The interaction of oppositely charged colloidal solutions have been extensively studied (Li *et al.*, 1994; Kabanov and Kabanov, 1995). However, most studies involved simple precipitation boundaries without the detailed studies of particles, as in the case of DNA and cationic

liposomes. Only a few electron microscopic studies of DNA–lipid complexes have been published (Sternberg *et al.*, 1994; Gustafsson *et al.*, 1995; Sterneberg, 1996). They report on undefined colloidal aggregates, occasionally surrounded by a halo of thin fibers. Detailed analyses established that spherical structures are aggregated liposomes and the thin fibers are lipid-coated DNA helices (Sternberg *et al.*, 1994; Sternberg, 1996). Figure 1.5 shows a freeze–fracture electron micrograph of such a particle.

Following these and other studies several models of DNA–lipid aggregates ('genosomes') have been postulated (Figure 1.6). So far none of these structures has been substantiated by more than one independent experiment. We can, however, divide the published structures into two groups: (1) dense condensed aggregates, and (2) heterogeneous systems containing spherical aggregates and fibrilar structures. The latter structures were observed predominantly in the 3,3[,V-,V',V'-

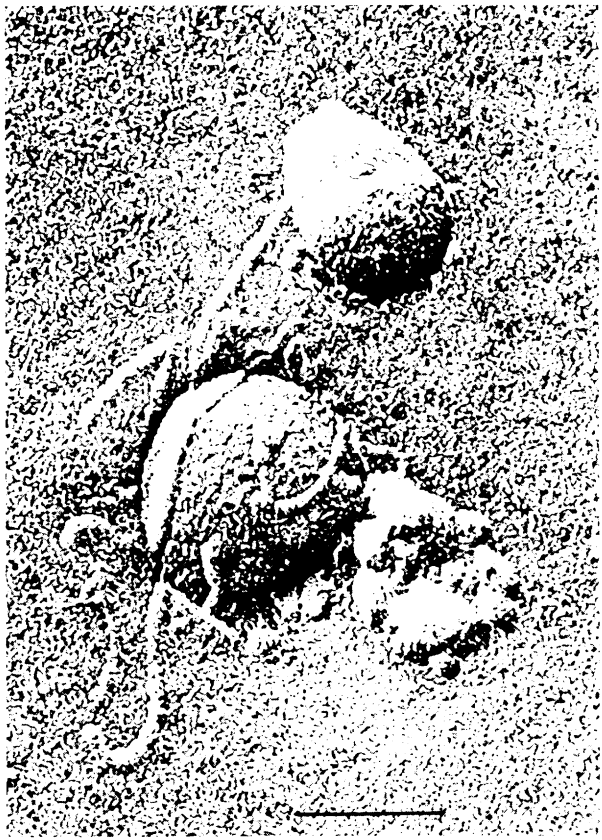

Figure 1.5 Freeze fracture micrograph of a DC-Chol DOPE(3:2) DNA complex. Globular aggregates and lipid coated fibrils can be seen. (Courtesy of B. Sternberg, Friedrich Shiller University, Jena, Germany.)

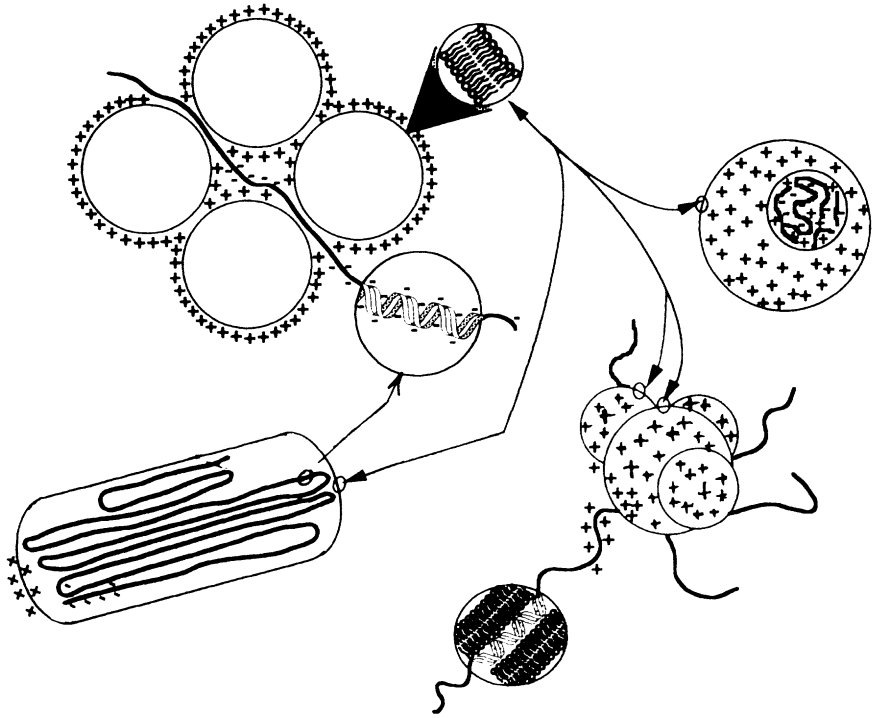

Figure 1.6 Schematic presentation of genosome models. Top left: stoichiometric model, in which DNA is adsorbed between cationic liposomes (Felgner and Ringold, 1989). Top right: cationic lipid coats condensed DNA (Behr, 1993). Bottom left: DNA induces liposome fusion, condenses, and becomes encapsulated in a lipid bilayer (Gershon *et al.*, 1993). Bottom right: aggregated liposomes with attached fibers of DNA encapsulated in a bilayer lipid cylinder (Sternberg *et al.*, 1994).

dimethylaminoethane-carbamoyl]cholesterol (DC-Chol) DOPE DNA systems and were shown to be DNA encapsulated into a tube of lipid (Sternberg *et al.*, 1994).

It is possible that the explanation of the appearance of those different structures is rather simple. DC-Chol cannot effectively condense DNA because, as a result of its pK value of approximately 6–7 (Zuidam and Barenholz, 1997), it has insufficient charge density at physiological conditions. Therefore, while the lipid coats DNA and DNA fibers aggregate some liposomes, the charge density is still not high enough to cause effective DNA condensation. Fibrils have also been seen with other monovalent cationic lipids such as *N*-[1-(2,3-dioleyloxy propyl]-*N*,*N*,*N*-trimethyl-amonium chloride (DOTMA) and 1,2-dimyristyloxypropyl-3-dimethyl-hydroxy-ethyl ammonium bromide (DMRIE), which are quaternary ammonium salts (Sternberg *et al.*, 1994). However, if complete neutralization and condensation occur, DNA tends to form dense aggregates which are often characterized by lamellar symmetry. Such structures were characterized by X-ray scattering and

high-resolution cryo-electron microsopy (Podgornik R, Frederik PM, Lasic DD unpublished data, 1994; Lasic *et al.*, 1996, 1997). Figure 1.7 shows a cryo-electron micrograph of a complex, which is characterized by intercalated lamellar symmetry. Recent work (Lasic *et al.*, 1996; Radler *et al.*, 1997; Lasic *et al.*, 1998) has shown that formation of these structures is a rather general phenomenon observed with a variety of cationic lipids and liposomes. Further, these complexes can be stabilized by replacing DOPE with cholesterol, condensation with spermidine, or inclusion of PEGylated phospholipids, while retaining high transfection activity *in vivo* (Liu *et al.*, 1996).

Condensed phases of (cationic) lipid bilayers and DNA belong to two different symmetry, as well as dimension, classes (Lasic *et al.*, 1997). Lipids condense into one of the several varieties characterized by either cubic, hexagonal, or smectic symmetry. Hexagonal mesophases of lipids are characterized by a 2D crystalline order perpendicular to the long axes of either lipid or water tubules, while smectic mesophases are characterized by a 1D quasi-crystalline order in the direction perpendicular to the bilayer surfaces and a 2D liquid order within the smectic layers. In the cases of lipid and DNA interactions the smectic-like lipid arrays 'clash' with the ordering tendencies of condensed DNA that prefers local hexagonal

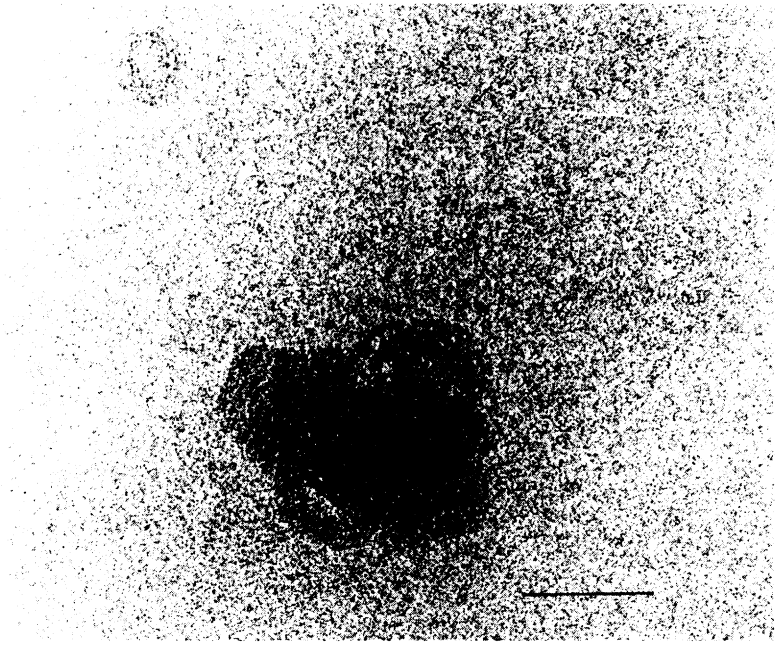

Figure 1.7 Cryo-electron micrograph of a dioctadecyldimethyl ammonium bromide : cholesterol (1:1) : DNA complex (charge ratio 2/1). The separation between lamellae is around 6.5 nm, which represents a lipid bilayer with DNA adsorbed in a single plane in 2D. (Courtesy of P. Frederik, Limburg University, Maastricht, The Netherlands.)

symmetry. It was recently established (Lasic *et al.*, 1997) that the result of these opposing ordering tendencies is the lowering of the effective dimension order of the condensed DNA phase. Instead of remaining in a 3D locally hexagonally packed line hexatic phase DNA becomes intercalated between smectic planes of lipid bilayers, so that DNA molecules are packed in essentially parallel domains. As a result the dimensional order changes from a 3D line hexatic into something that shows roughly a 2D smectic symmetry. Figure 1.8 shows a molecular graphics

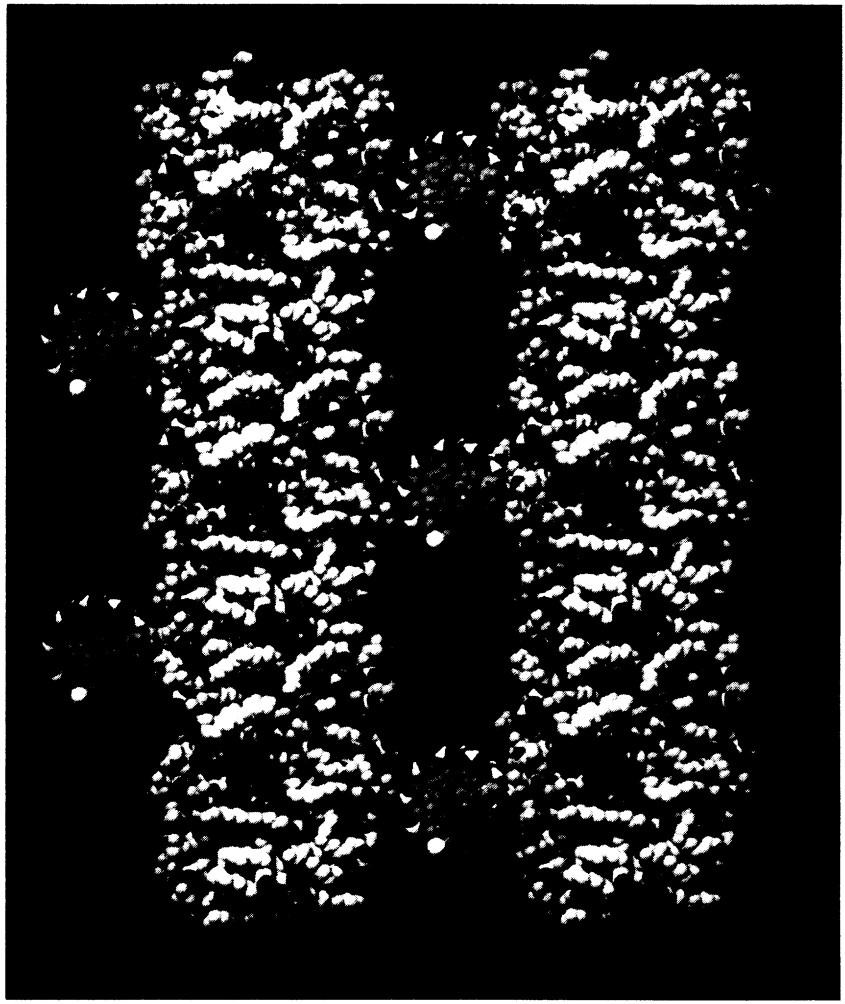

Figure 1.8 Structure of the DNA and cationic lipid complex (Lasic *et al.*, 1997). DNA is intercalated between smectic planes composed of lipid bilayers. Bilayers form a lamellar structure. The 2D condensed DNA is adsorbed and sandwiched between the bilayers. (Courtesy of M. Hodoscek, National Institute of Chemistry, Ljubljana, Slovenia.)

representation of an intercalated lamellar phase consisting of smectic bilayers with sandwiched 2D condensed DNA. As in the case of lipid bilayers, this 2D smectic order is expected to show a 1D quasi-crystalline order perpendicular to the long axes of the DNA molecules, which is not observed for a 3D smectic order. This line of thought is reasonably well borne out by the results of experiments, suggesting that the intercalated DNA contributes an additional peak to the scattering function of the lipid–DNA aggregates.

The electrostatic interactions between the DNA and cationic lipids evidently contribute to the effective lowering of the dimension order of the condensed DNA phase interacting with the smectic ordered cationic lipid arrays. In this case, the energy of the electrostatic binding of oppositely charged DNA and lipid molecules has to be accounted for along with the symmetry considerations for the combined ordering of DNA and lipids. Intercalation of DNA in this perspective serves two basic purposes. First, DNA condenses into an ordered phase, which looks, except for the lower dimension order, quite similar to other condensed forms of DNA (e.g. condensed via osmotic pressure or simple multivalent counterions). Second, the large negative charge of DNA effectively neutralizes the repulsive forces between positively charged lipid bilayers in the smectic array and thus stabilizes the complex. Therefore, neutralizing the destabilizing electrostatic effects in the lipid array proper apparently compensates the lowering of the dimension order of the DNA within the lipid array. This quid pro quo actually contains some additional details in terms of the balance of forces that are described below.

As discussed in the previous section, the elastic shape fluctuations of the DNA molecules determine the bulk of the free energy in the cholesteric phase, where DNA still retains (although very locally) an hexagonal order (Strey *et al.*, 1998). Similar forces also act between molecules in 2D sheets intercalated between lipid bilayers in the lipid–DNA complex. The presence of strong elastic fluctuations can be ascertained from the width of the DNA diffraction peak in the complex, as well as from the conformational snapshots provided by the strong adsorption of DNA to mica-immobilized cationic lipid bilayers (Fang and Jie, 1997). Elastic shape fluctuations of the DNA molecules within the intercalation planes result in an entropy contribution to the free energy of the system that force DNA chains apart. However, this tendency to lower the density of intercalated DNA is offset by the electrostatic attraction between the positive charges of lipids and negative charges in the DNA backbone that tend to increase the density of intercalated DNA. It is unclear at this point whether Helfrich-like interactions (Lipowsky 1995) between the fluctuating lipid bilayers would contribute to the overall energy balance. It is more probable that lipid bilayers with intercalated DNA fluctuate only through collective undulations preserving the separation between the bilayers set effectively by the diameter of the DNA. Also the ordered state of intercalated DNA would invariably lead to a direction-dependent curvature elastic constant of the layers, since it is easier to bend the bilayer with adsorbed ordered DNA in the direction perpendicular to the DNA axis.

DNA is, of course, a chiral molecule because of its helical structure (de Gennes and Prost, 1993). This means that at sufficiently high densities DNA tends to form a cholesteric liquid crystalline phase (Livolant and Leforestier, 1997). These DNA densities are comparable to the densities of DNA in the genosome (Lasic *et al.*, 1997; Radler *et al.*, 1997). However, no cholesteric textures have been observed for genosomes under crossed polarizers. It appears that chiral interactions are strong enough to induce cholesteric order in bulk DNA but not in DNA intercalated between multilamellar lipid bilayers. Such a system would show characteristics of a smectic with an in-plane elastic stiffness tensor that would rotate like a cholesteric director. It remains to be seen exactly how chiral interactions between DNA molecules couple to the smectic lipid order in genosomes (Podgornik and Zeks, 1997).

The above analysis obviously applies to a thermodynamically stable end-state of the DNA cationic lipid complexes. There are probably many dynamically constrained precursor states that form during interaction of DNA with cationic liposomes. In such systems, strong interactions between the DNA and liposome surface could result in opening up the closed lipid bilayers yielding some new multilamellar arrangements with intercalated DNA. At present the attempts to identify those possibly numerous nonequilibrium states, which precede the final intercalated multilamellar arrangement, do not go far beyond speculation. However, those structures probably represent relatively stable kinetic 'traps.' In practice, DNA–lipid complexes can be stable in liquid form for months and in freeze-dried form for years.

In conclusion, lipids and DNA exhibit rich polymorphism, which is also reflected in (and governs) their interactions. Furthermore, it is this polymorphism which allows the production lipid–DNA systems effective for gene delivery. While many researchers are trying to improve transfection by synthesizing novel cationic lipids, we believe that the control over colloidal properties of DNA–lipid complexes is at least as important as the synthesis of new molecules. This claim is strengthened by the fact that despite a decade of work no clear molecular structure–transfection activity correlation have been found for lipid-based transfection systems, especially for *in vivo* applications. Furthermore, in some cases enhanced tansfection can be related simply to the detergent activity of the lipids. On the other hand, some clear colloidal structure–transfection activity relationships have been observed with lipid-based transfection (Lasic, 1997; Hong *et al.*, 1997; Templeton *et al.*, 1997). In this chapter we tried to present and discuss colloidal properties of lipids, DNA, and their complexes, which are a consequence of various attractive and repulsive interactions as well as liquid crystalline behavior of both reactants. We believe that the improved understanding of the thermodynamics and kinetics of these complexes will contribute significantly to the design of transfection delivery vehicles with improved properties.

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